The Circular Dichroism of Variants of β-Lactoglobulin

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SUMMARY

The circular dichroism of variants of β-lactoglobulin has been measured and the over-all differences between the species were found to be small. The dichroic spectrum given by the aromatic residues in β-lactoglobulin B is shown to be decomposable into five negative dichroic peaks. These can be correlated with various types of chromophore residues, with the two strongest peaks, at 293 and 284 nm, being probably due to tryptophan.

Changes in the spectrum, obtained as β-lactoglobulin is denatured by alkali and acidic methanol, are shown. The native protein has a negative dichroic peak in the vicinity of 215 to 220 nm, and with increasing pH, this peak shifts downward toward 201 nm and increases 4-fold in intensity. The positive dichroic peak at 196, seen in the native protein at pH values below 5, also is shifted to shorter wave lengths without, however, changing greatly in intensity. Acidic methanol, on the other hand, causes a conversion to a spectrum qualitatively like that of an α-helix, with negative maxima at 222 and 207 nm and a positive extremum at 193 nm. These changes are discussed in the light of the circular dichroic spectra of polypeptides in known conformations.

The relationship between secondary structure in the interior of a protein molecule and the functionality of groups on its surface is currently the subject of much work. A large amount of such data has been gathered in recent years by means of the study of optical rotatory dispersion and circular dichroism (1, 2). Under proper conditions, these techniques are able to identify various types of secondary polypeptide structure, such as the α-helix (3), the random or unordered structure,[1] and the various β or pleated sheet structures, both in solution (4–8) and the solid state (8, 9). The ability of these techniques to make such discriminations is of interest, particularly since it is recognized now that, in many proteins, a number of peptide chain conformations probably coexist and must be taken into account (7, 10, 11).

Quite recently, with the availability of commercial instruments capable of detecting and recording CD[2] in the ultraviolet range down to 185 nm, it has become possible to make this kind of measurement on proteins and polypeptides, and, even though no information can be obtained from CD that, in theory, cannot be obtained from ORD (2), the bands occurring in the CD spectrum are more discrete. This narrow band character has the practical advantage of enabling the contribution of chromophores, which may be active in closely spaced regions of the spectrum, to be more easily resolved and, hopefully, identified. CD is also relatively free of some of the problems that arise in attempting to recognize small Cotton effects. Such effects may be given by aromatic or disulfide side chains (12–15) and be masked by superposition on the much stronger levorotations possessed by proteins in the region of 250 to 280 nm, which are caused by transitions at lower wave lengths; they may also arise from very weak transitions in single chromophores having other strong transitions (16) and thus be hidden in the background rotation of the strong bands.

The present work presents data on the CD spectrum of bovine β-lactoglobulin. This protein occurs in two common genetic variants (17); two much rarer variants (18) are also known at present.[3] The pH dependence of the CD spectra of the A and B variants (β-A and β-B) has been studied in detail and compared at certain conditions with the C variant and a preparation of goat β-lactoglobulin (G-β). Previously reported conformational analyses (11) of the native proteins have been confirmed, and the optically measurable difference between the varieties are seen to be small.

EXPERIMENTAL PROCEDURE

Native bovine proteins were multiply recrystallized samples of three genetic variants prepared by us from the milk of homozygous cows. The G-β was prepared from herd goat milk by a procedure to be described elsewhere.[4] Aqueous stock solutions

of the proteins were prepared at approximate concentrations of 5 g per liter by dissolving a lyophilized crystal slurry in 0.10 M NaCl or NaF and subsequently adjusting the pH with 1 M NaOH or HCl. Stock solutions were then clarified in a table-top centrifuge and concentrations determined spectrophotometrically with an absorptivity[5] at 278 nm of 9.6 dl per g-cm. CD and ORD measurements were made on a Durrum-Jasco ORD UV-5 apparatus[6] with CD attachment and some ORD measurements on a Cary model 60 instrument. pH was measured on a Beckman model G meter, and a Zeiss PMQ II spectrophotometer was used for concentration analyses. Quartz cells used for CD experiments were a demountable 1-cm cell, a 1-mm cell with fused on windows, and a demountable cell with a 0.040-inch (0.11-mm) plastic spacer. The same spacer was used for the entire series of runs. All CD spectra were run at least twice on the same cell filling, and base line runs were made as soon before or after the sample run as possible. Solutions of proteins in methanol were prepared by diluting concentrated aqueous stock solutions (50 g per liter at pH 2) into acidic methanol (0.01 ml of concentrated HCl up to 10.0 ml with anhydrous methanol).

Ellipticities $[\Theta]_\lambda$ in units of deg cm² dmole⁻¹ were calculated from CD spectral tracings by the following equation:

$$[\Theta] = \frac{3300}{lc} \times \text{scale setting} \times \text{reading} \qquad (1)$$

where l is optical path in centimeters, c is concentration in decimoles of residues per liter, "scale setting" is degrees for 10-cm deflection on the Jasco instrument (only 0.005 and 0.002 were used in this work), and "reading" is centimeter deflection from base line of the CD tracing at wave length λ. The mean residue weight of the β-lactoglobulins was taken to be 112 as previously (20).

Theoretical ORD curves were calculated from CD spectra via the Kronig-Kramers transform which has the form (21):

$$[m'(\lambda)]_k = \frac{2}{\pi} \int_0^\infty [\theta'(\lambda')]_k \frac{\lambda'}{\lambda^2 - \lambda'^2} \, d\lambda' \qquad (2)$$

Primes indicate that the Lorentz factor $3/(n^2 + 2)$ has been applied and $[\Theta']_k$ and $[m']_k$ are the corrected mean residue ellipticity and rotation, respectively, for the k'th band in deg cm² per dmole units.

If the assumption is made that the k'th CD band is gaussian with respect to wave length,

$$[\theta] = [\theta^0]_k e^{-x^2} \qquad (3)$$

where $x = (\lambda - \lambda^0_k)/\Delta^0_k$, and that the peak, centered at λ^0_k, has a height in ellipticity units of Θ^0_k and a width, in wave length units, of $2\Delta^0_k$ at height $1/e(\Theta^0_k)$, then the following equation may be obtained, after Moscowitz (22):

$$[m'(\lambda)]_k = \frac{3}{n^2 + 2} \frac{2[\theta^0]_k}{\sqrt{\pi}} \left(e^{-x^2} \int_0^x e^{t^2} \, dt - \frac{\Delta^0_k}{2(\lambda - \lambda^0_k)} \right) \qquad (4)$$

This form is recognized as a modified Dawson's integral, for which calculated tables are readily available. These tables are read into the computer and, by the use of a relatively simple

[5] The G-β has an absorptivity equal to that of the bovine proteins within experimental error (Footnote 4).

[6] Mention of specific products or manufacturers does not imply endorsement by the United States Department of Agriculture to the detriment of others in the same business.

"bookkeeping program," the transform for each dichroic band is obtained at each desired wave length. The over-all rotatory dispersion plot is then the sum of the individual transformed bands at each wave length.

RESULTS AND DISCUSSION

In order to verify proper operation of the instrument, the CD spectra of solutions of poly-L-glutamic acid, insulin, and L-cystine were measured over the far ultraviolet spectral range. The poly-L-glutamic acid spectrum, taken at pH 4.3 and in a 0.1 M NaF solvent, agrees with data published by Holzwarth and Doty (23), Velluz and Legrand (24), and Cassim and Yang (25) and with theoretical predictions of the optical activity of the α-helix (26); this spectrum is characterized by two well defined and approximately equal ($[\Theta] \approx -4 \times 10^4$) negative extrema at 207 and 221 nm and a crossover point at 201 nm. The CD spectrum of insulin at pH 8.4 also agreed well with the 320- to 220-nm data of Beychok (15). Our measurements show a small but definite indication that the 275-nm maximum is split: it seems to consist of two negative CD bands, both broad, with peaks close to 272 and 279 nm. The data at lower wave lengths show unmistakably the two α-helical extrema at 207 and 221 nm ($[\Theta] \approx -11 \times 10^2$), an indication that a significant contributor to the native structure of insulin is this conformation. This fact is in agreement with the literature, where reported values vary between 30% α-helix (3), based on ORD data, and 66%, based on hyperchromicity studies (27). The CD spectrum of L-cystine was found to be in good agreement with the data of both Beychok (15) and of Velluz and Legrand (24).

β-Lactoglobulin

The CD spectra of the β-lactoglobulins may be conveniently divided into two spectral ranges, from 350 to 260 nm and from 260 nm to optical cutoff, which, in the present instrument, can be as far down as 183 nm in the thinnest cell, if solvent allows.

A. 350 to 260 nm—In this upper wave length range, the ellipticities measured are typically two orders of magnitude smaller than those seen in the range below 250 nm. The results given by β-lactoglobulin from 330 to 250 nm are shown in Fig. 1.

Part A shows, as the *solid line*, the experimental CD spectrum which has three unmistakable peaks at 293, 285, and 277 nm and seems to indicate additional weak dichroic activity near 270 nm. This line is the average of six experiments with overlapping concentrations and pH values from 1.5 to 5, over which pH range no significant or reproducible change in the spectrum can be seen. (All the species of β-lactoglobulin examined give highly similar spectra in this range.) No fewer than four runs are averaged at any single wave length point, which are picked off the instrumental tracing every nanometer except for the flat leading and trailing edges of the curve. There is undoubtedly additional negative dichroic activity present in the wave length region near 260 nm, as the signal never quite reaches the base line at these pH values, although it does so at pH 11 and above. It seems significant that this is the spectral range of the negative CD signal given by cystine (15).

The spectrum of Fig. 1A was divided, by trial and error, into five symmetrical bands, shown as the *dashed lines*, which account for close to 90% of the area between 330 and 260 nm (the area not divided is mostly in the suspected "disulfide" region) and the Kronig-Kramers transform was applied. The sum of the

five derived Cotton effects is shown in Fig. 1B. In this form, however, the calculated ORD cannot be compared directly with experiment since the absolute rotation is rapidly increasing toward lower wave length as a result of the large negative Cotton effects peaking in the 220- to 230-nm region.

Fig. 1C shows the calculated rotatory dispersion curve (dashed line) which has been obtained as follows. The measured values of the rotation (11) in the regions 250 to 265 nm and 300 to 320 nm were corrected (see below) for the total calculated contributions of the aromatic Cotton effects. The corrected values were plotted and joined in a single smooth curve through the 265- to 300-nm range. The results of the Kronig-Kramers transform (Curve B) were then added point by point to this base line and the sum was plotted as the broken line in part C. The solid line in C is the plot of the previously measured experimental values (11). The agreement is quite satisfactory, an indication that the division of the CD curve (dashed lines, part A) is not greatly in error, and that the construction of the smooth base line through the optically active region of tryptophan and tyrosine absorption is reasonable.

Examination of Fig. 1 points up the important advantage of the CD technique: here optical activity manifests itself as four or five small dichroic peaks (Fig. 1A). In ORD these separate activities are overshadowed by the rapidly increasing background rotation, with the result that the ORD curve gives the appearance of having either two negative or one positive Cotton effect superimposed on it (Fig. 1C). ORD peaks and troughs in similar positions have been observed also in α-lactalbumin (28) and human carbonic anhydrase (29); in both cases these have
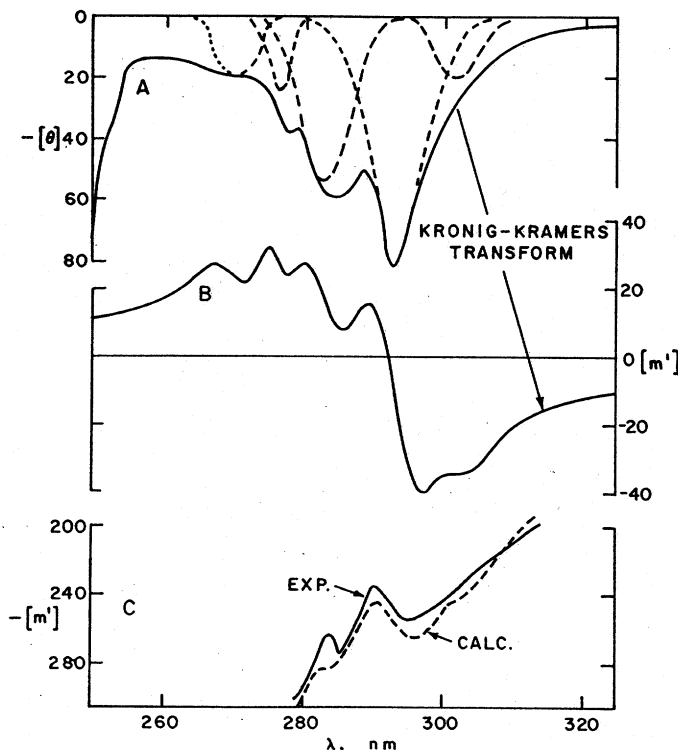

FIG. 1. A, ——, circular dichroic spectrum of β-lactoglobulin B; – – –, empirical decomposition. B, dispersion of optical rotation calculated by Kronig-Kramers transforms of decomposed CD spectrum. C, ——, experimental ORD spectrum; – – –, curve of part B added to smooth base line obtained as described in text.
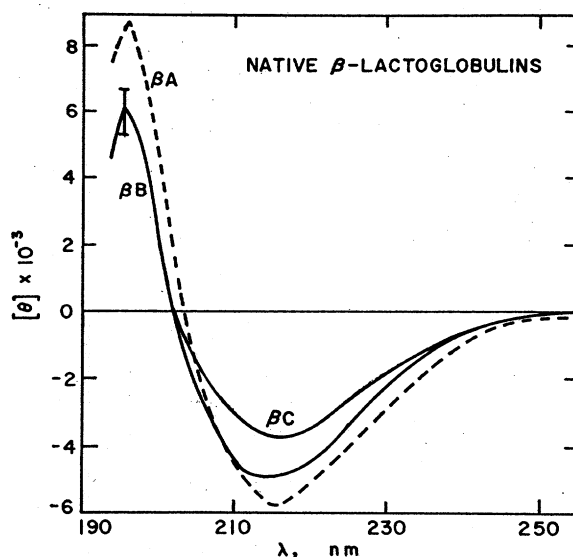

FIG. 2. Circular dichroic spectra of three varieties of bovine β-lactoglobulin in 0.1 M NaCl, pH <5.2.

been ascribed to tryptophan residues. In this connection, it seems significant that Rickli et al. (30) report an acid difference spectrum of carbonic anhydrase B and find two strong absorption peaks, located at 292 and 285 nm (with the 292 peak being the most intense of the pair), which they relate to tryptophan residues. Such an assignment seems reasonable also for the β-lactoglobulin case, since, as can be seen in Fig. 1, these positions coincide with the two strongest CD bands, at 293 and 285 nm.

The two negative dichroic bands between 265 and 280 nm may possibly be due to tyrosine residues, in view of the ORD data reported by Glazer and Simmons on ribonuclease (31). This tryptophan-free protein, in its native state, shows an apparently well defined negative Cotton effect with a midpoint a little below 280 nm. The same effect has been seen at 282 nm in cytochrome c and ascribed to tyrosine (32), and furthermore, the CD of ribonuclease displays broad negative dichroism with a maximum near 275 nm (15). Our division of the β-B CD spectrum, while admittedly empirical, yields two negative bands of approximately equal size, centered at about 270 and 277 nm. Our CD spectrum of insulin also shows what appears to be the beginning of a split into two bands of equal intensity which peak somewhere near these wave lengths. Beychok has indicated, however, that in this protein optical activity in the vicinity of this wave length may be related to cystine residues (15), so that at present no completely unequivocal assignment may be made.

The signs of the optically active bands observed in this spectral region seem worthy of comment. The CD spectrum of β-lactoglobulin above 260 nm is composed of at least five negative bands. In this respect, the situation is similar to that of two varieties of human carbonic anhydrase (33), ribonuclease (15) and α-lactalbumin.[7] Lysozyme (15, 34) and carboxypeptidase A,[8] however, display only positive dichroism in this region, while chymotrypsin and chymotrypsinogen (35) show negative dichroism above 300 nm and positive dichroism between 280 and 300 nm. In what seems to be a unique case at present, a positive CD peak at 246 nm in the spectrum of carbonic anhydrase C

[7] R. Townend and M. J. Kronman, unpublished observations.
[8] S. N. Timasheff and L. Stevens, unpublished observations.

(which gives only negative dichroism above 260 nm) has been related to tyrosine residues (33). The fact that only positive Cotton effects have been seen in ORD studies in L-tryptophan, poly-L-tryptophan (13), tyrosine (12, 14, 36), and poly-L-tyrosine (14) and only positive CD bands in poly-L-tyrosine solutions (37) and poly-L-tryptophan films[9] emphasizes the extent to which specific secondary and tertiary structures, which impart asymmetric orientation to aromatic side chains, can influence the optically active transitions of these groups.

B. 260 nm and Below—Fig. 2 shows the CD spectrum of native β-A, β-B, and β-C down to 194 to 195 nm. The curves are of the same general shape, showing a broad, unsymmetrical negative extremum near 215 nm, a crossover at 202 to 203, and a positive peak at 196 nm. The β-A curve (*dashed line*) is the average of six runs, the *lower solid curve* (β-B) is the average of three, and the *upper solid line* (β-C) is a single run at pH 4.5. The averages are taken over the pH range from 1.0 to 5.6. The difference in the magnitude of the peaks given by the two common β-lactoglobulins seems to be real, particularly at the 196 peak. The G-β variety gives a curve practically superimposable on that of β-B in this range of pH and wave length.

Fig. 3 shows the CD spectra of β-B as a function of pH. The curve shown in Fig. 1 is reproduced here as the native protein (pH 1 to 5) curve. In the pH range of 5 to 9, two pH-dependent reversible conformational transitions are known to take place (38, 39), both of which result in changes in the optical rotatory parameters and in the number of titratable groups exposed to solvent. Between pH 9.5 and 10.5, no drastic change in the CD spectrum is seen, as shown by the *curve labeled pH 10* (average of four runs, pH 9.5 to 10.5), in agreement with the near constancy of the a_0 parameter of β-B in the same range (39). In β-A, where no such plateau in a_0 is found, the CD spectra progress continuously above pH 9 (40). Around pH 11 further large changes in optical properties occur as a result of quite small changes in pH, as can be seen by comparison of the next three curves, pH 11.2, 12, and 13, respectively. (This same phenomenon is evident in Fig. 1 of Reference 11, where a large change in the depth of the Cotton trough at 216 nm is seen to be caused by a change in pH from 11.3 to 11.6.) A noteworthy feature in this family of curves is the change seen near 217 nm. No negative CD band is resolved when the protein is below pH 5, but one begins to be visible as a shoulder above pH 9, is seen as a rather well separated bimodality at pH 11, and diminishes again at pH 12 and above.

The pH 13 curve presents certain experimental difficulties. The solution the spectrum of which is shown was prepared by dissolving lyophilized crystalline protein in 0.1 M NaOH. It is necessary to measure the CD within a few minutes, as the optical activity shortly begins to increase at 200 nm and the shoulder at 217 to 218 begins to disappear. (After several hours at 25°, the optical activity at all wave lengths has decreased drastically, possibly because of alkaline hydrolysis of the protein, and there is an appearance of a yellow color in the solution.) The optical density of the 0.11-mm cell filled with 0.1 M NaOH is about 4 at 197 nm and reaches the same value at 198 nm with a 0.7 g per liter lactoglobulin solution. As a result, the apparent upturn at 202 nm shown in Fig. 3 for the pH 13 solution is somewhat uncertain, although it seems to be present in freshly prepared solutions; this feature cannot be seen if the solutions are 1 hour

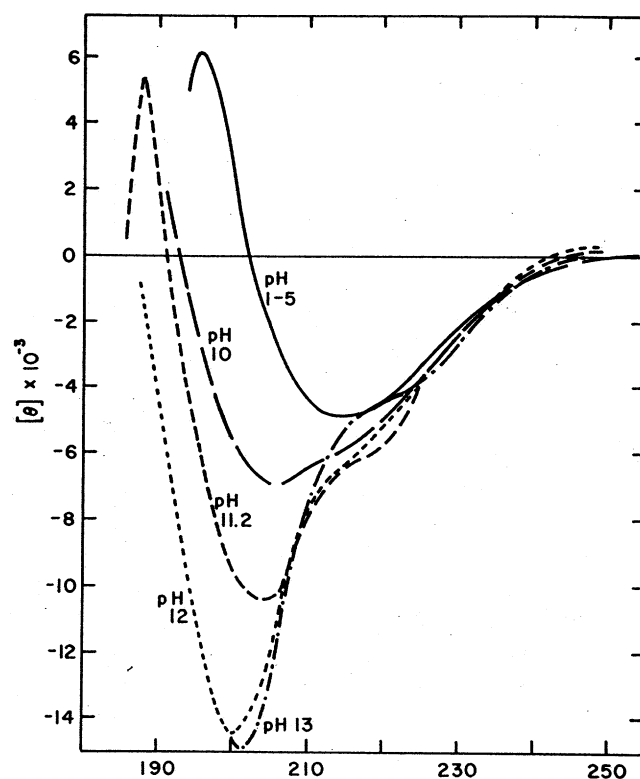[9] L. Stevens, S. N. Timasheff, R. Townend, and G. D. Fasman, to be published.

Fig. 3. Circular dichroic spectra given by β-lactoglobulin B as a function of pH in 0.1 ionic strength NaCl-NaOH solutions.

old. In this family of curves (Fig. 3), the intensity increases and blue shift occurs in the maximum as pH is increased. This parallels the increase of $(\alpha)_\lambda$ and a_0, seen generally upon alkaline denaturation of proteins, and of the β-lactoglobulins in particular. The positive CD band, located at 196 nm in the native protein, also seems to shift downward in wave length under more alkaline conditions, but, because of the large absorptivity of the hydroxide ion, it is impossible to observe at pH 12 with present instruments and light sources.

β-Lactoglobulin B may be described qualitatively (11) as having a small amount (10% or less) of α-helical regions, the remainder being about equally divided between unordered[10] and β structures. Infrared spectral studies (41) have also established that the β structure is quite probably of the antiparallel type. This type of β structure has been shown to have a CD spectrum with two well defined bands, a negative one peaking at 217 nm and a stronger positive one with a peak at 196 nm (5, 6, 42). Unordered structures so far examined have strong negative dichroism peaking at approximately 195 nm (5, 6, 23, 24), a weak positive band at 220, and a third extremely weak negative one near 235 nm. Thus, in native β-lactoglobulin, the spectrum around 190 to 200 nm is dominated by the strong positive contributions of the α-helical and β-structured components present. If the primary effect of increasing pH is the conversion of ordered

[10] For a globular protein molecule in the native state, the term "unordered" structure should be used in preference to "random" structure. The latter implies constantly changing positions of segments of the polypeptide chain in the usual polymer statistics sense. In globular proteins, these positions are fixed in space, even though there may be successions of amino acid residues and peptide bonds which lack any regularly repeating pattern of mutual orientation.
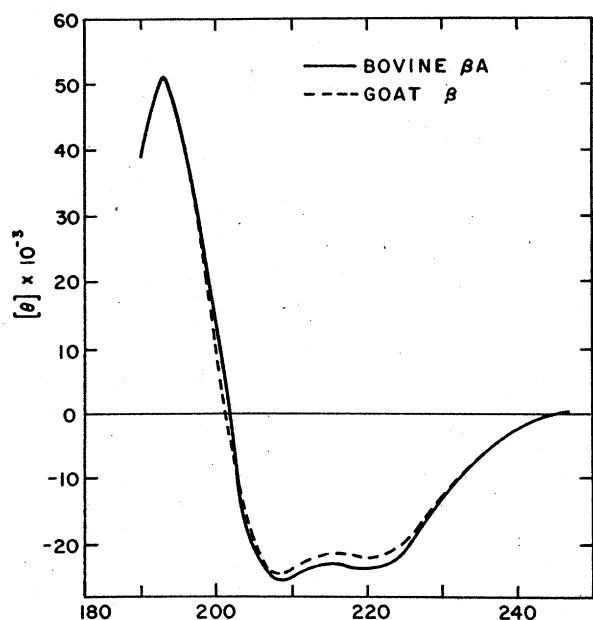
FIG. 4. Circular dichroic spectra of β-lactoglobulin A and the β-lactoglobulin of goats' milk in 99% (by volume) methanol, 0.01 M, in HCl.

into unordered structures, as would be expected, this family of spectra can be readily understood. The native protein (pH 1 to 5) shows no CD peaks in the 210- to 225-nm range that can be unequivocally identified with any particular conformation. At pH 10 and above, however, one can see increasingly the tendency of the spectrum to be dominated by that of the unordered structure and to approach gradually the negative maximum at 195 nm. In this pH range, too, the shoulder near 217 becomes apparent. Net negative dichroism at this wave length, where unordered structures have positive dichroism, suggests a considerable contribution from some other structure, possibly a β structure. This could result either from a native core more resistant to alkaline denaturation than other portions of the molecule or to reaggregation of denatured protein into a β form. In this connection, it seems relevant that none of the proteins examined by us in a denatured state exhibit the positive CD band at 220 nm[8]; negative dichroism between 217 and 230 nm is the common observation. In fact, α_s-casein, which is believed to be structureless, has a prominent negative dichroic shoulder in this region (40).

When β-B and β-A are denatured by increasing concentrations of acidic methanol, a small amount of β structure seems to persist at 99% concentration of nonaqueous solvent (11). Under these conditions, the molar rotations at the 199- and 233-nm ORD maxima are about 60% of that which can be obtained with homopolymers in such helix-forming solvents. This is to be expected in a nonreduced protein because of constraints imposed by disulfide bonds and other steric factors. Upon destruction of the S—S bonds by —S— sulfonation, dissolution in the acidic methanol results in almost total conversion to the α-helical structure (11). Fig. 4 shows the CD patterns of two β-lactoglobulin varieties, β-A and the goat protein G-β in 99% MeOH, 0.01 M in HCl. The amino acid differences between the two proteins, while significant,[4] do not involve the cystine or proline contents, and their dichroic spectra are seen to be essentially superimposable. The positions of the CD maxima in this figure

are exactly those of the α-helix (23, 24, 5, 6): two negative peaks at 207 and 222 nm, with approximately equal ellipticities ([Θ] $\cong$ -2.5×10^4), a crossover point at 200 nm, and a positive band peaking at 190.5 ([Θ] $\cong 5 \times 10^4$). With the use of the ellipticities of extrema given by poly-L-lysine (5, 6) and poly-L-glutamic acid (23, 24) as models, under conditions in which the latter may be assumed to be maximally helical, the β-A and G-β may be estimated to contain 65 $\pm$ 8% of the α-helical conformation in acidic methanol. This is in quite reasonable agreement with the value of $\approx 60\%$ estimated from the depth of Cotton effect troughs (11).

Compositional Analysis of β-Lactoglobulin B—At this point, it is tempting to estimate the relative amounts of the three kinds of structures existing in the native protein. Such an estimate has been made previously (11) with a_0 and b_0 values obtained from rotations measured in the visible region of the spectrum. However, it has been pointed out (14, 28, 39) that Cotton effects due to aromatic chromophores may strongly effect the b_0 and [m'] values obtained at higher wave lengths. Availability of the circular dichroic spectra in the region of this chromophore absorption yields a way to circumvent this difficulty. When the CD spectrum (Fig. 1A) is decomposed and subjected to the Kronig-Kramers transform, rotation data are produced which may be extended as far as desired into the visible region of the spectrum. These numerical values may then be subtracted from the accurately known rotations at the major wave lengths of the mercury arc (20), and truer Moffitt-Yang (43) parameters may be calculated from the corrected data.[11] In an earlier study (20), a practice was followed of plotting the Moffitt-Yang points as fairly large circles and drawing a straight line touching them all (579 to 313 nm). Although this procedure was internally consistent and capable of following relative changes in internal structure,[12] intrinsically incorrect a_0 and b_0 values were obtained. The calculations were repeated with the same raw data after subtraction of the aromatic contributions. As a result, the slight concavity, which has always been observed in Moffitt-Yang plots of rotation data taken on native β-lactoglobulin (44, 38, 20), was substantially decreased and the straight line fit greatly improved. The a_0 parameter, which is obtained from an extrapolation to infinite wave length, was decreased only slightly, from -160 to -155 deg cm^2 $-$ dmole^{-1} for β-B. In the case of the b_0 parameter, the situation is quite different. This number is given by the slope of the Moffitt-Yang plot, of which the lower wave length points are affected more strongly by the aromatic Cotton effects; for β-B, this value was reduced by 15%, from -72 to -62 deg cm^2 $-$ dmole^{-1}. When the correct value of these parameters is known, one can set up simultaneous equations following Urnes and Doty (3).

$$b_0 = \alpha(b_0)_\alpha + \beta(b_0)_\beta + u(b_0)_u$$
$$a_0 = \alpha(a_0)_\alpha + \beta(a_0)_\beta + u(a_0)_u \tag{5}$$

[11] Similar corrections should be made for the contributions of all other bands not related to peptide bond transitions, such as aromatic bands at lower wave length. In many cases, and particularly so in β-lactoglobulin, these are too weak to be resolved, so that such a correction cannot be made. This omission should not result in significant errors in this case, since the wave lengths of interest are far removed from these bands. In some cases, however, where these bands are strong (33), resultant errors in b_0 and a_0 may be considerable.

[12] Small errors in following such conformational changes could occur if the conformational change were accompanied by changes in specific side chain CD bands.

Here α is the fraction of the molecule in the α-helical form, u the fraction in the unordered form, and β is that in the pleated sheet (β) conformation $(\alpha + \beta + u = 1)$.

When the most likely values of $a_0(\alpha) = 0$, $b_0(\alpha) = -630$, $a_0(u) = -650$, $b_0(u) = 0$, and the values of Ikeda, Maeda, and Isemura (8) for the β structure, $(a_0(\beta) = +400$, $b_0(\beta) \doteq 0)$, are applied to Equation 5, and with the use of the experimental values for β-B corrected as explained above, one obtains a compositional analysis of 10% α-helix, 47% unordered structure, and 43% as presumably antiparallel β structure. The values assumed here for the a_0 and b_0 of the β structure are subject to much more uncertainty than comparable values for the α-helical and unordered conformations. However, it seems probable that $b_0(\beta)$ is very small; Volkenstein and Zubkov (45) have recently calculated a b_0 of close to zero for a pleated sheet structure via a quantum mechanical method. The use of Equation 5 may be questioned because of the known sensitivity of a_0 to the nature of the solvent (46). In the present study, however, values for the three conformations have been systematically varied within the broad limits assigned to them (16); the resulting changes in the conformational analysis are surprisingly not large enough to affect the over-all qualitative conclusion.

In order to check whether this analysis is consistent with the CD and ORD spectra obtained, the CD spectrum was first decomposed by subtracting proportional contributions of α-helical and unordered forms from the native protein spectrum. Poly-L-lysine was used as a model; 10% of its ellipticity at pH 11.2 and 47% of its ellipticity at pH 7 (5) were subtracted from the ellipticity of the protein at selected wave lengths. An analogous procedure was followed for the ORD spectrum. The individual curves and the resultant remainder curves are shown in Fig. 5. On the left, the CD of native β-B (pH 1 to 5) is presented as the short dashes; 47% of pH 7 poly-L-lysine is shown as the long dashes. On this curve, one sees the strong negative peak centered at 195 nm and the weaker positive peak near 218 nm. (Not shown on this figure because of the limitations of scale is the weak ($[\theta] \simeq -20$) negative dichroic peak seen in unordered poly-L-lysine at around 238 nm (40).) Ten per cent of the molar contribution of the α-helical (pH 12) poly-L-lysine is shown as the dotted line; when this curve and the u structure are subtracted from the CD spectrum of the native β-B, the solid line remainder curve is obtained. This curve is characterized by a positive peak at 196 nm and a negative one at 217 nm, the exact peak positions seen in the CD spectrum of the antiparallel β structure of poly-L-lysine (5, 6) and of solubilized silk fibroin (42).

This curve was divided graphically into two curves, centering at 196 and 217 nm, and the Kronig-Kramers transform was applied. The resultant rotational data are plotted as the circles on the right-hand portion of Fig. 5. The solid line is the ORD remainder curve. The agreement is quite reasonable and indicates internal consistency between the CD and two types of ORD analysis. Furthermore, it supports the conclusion drawn from infrared spectroscopy that β-lactoglobulin contains antiparallel β conformation (41).

Examination of the remainder curve of Fig. 5 reveals that the ratio of the ellipticities of the 196-nm positive peak to the 217-nm negative peak is nearly 6:1. In aqueous solution at pH 12, antiparallel β structured poly-L-lysine (5, 6) has a comparable ratio of 1.5:1, but, when the same poly-L-lysine at pH 7 is converted to β structure by 0.12 M sodium dodecyl sulfate (6, 40), the positive peak has an intermediate value of approximately
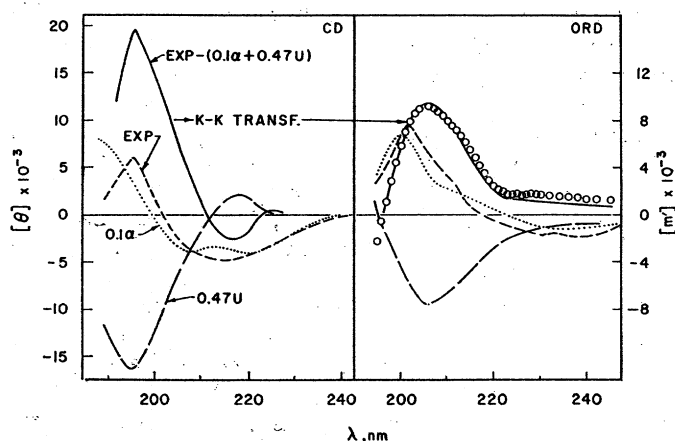

Fig. 5. Experimental circular dichroic spectra (left) and optical rotatory dispersion spectra (right) of β-lactoglobulin B and proportional fractions of poly-L-lysine spectra used as models for α-helical and unordered structures. The circles represent the results of the Kronig-Kramers transform (k-k Transf.) applied to the CD remainder spectrum as described in the text.

3.5 times the ellipticity of the negative peak. The identical effect of increase in the positive CD peak at the expense of the negative one has been demonstrated also in β structured silk fibroin as the methanol content of the dispersing medium is increased (42). These reports indicate that the dichroic bands characteristic of at least some kinds of β structure are quite susceptible to solvent effects, in particular, to the polarity of the immediate environment. The relative intensities of the two bands of the remainder CD curve would thus seem to be quite consistent with the location of the β conformation within the nonpolar interior of the protein. A somewhat similar effect of enhancement of certain CD bands with minimal effect upon others has been shown by Glazer and Simmons (34) to occur in lysozyme upon addition of ethylene glycol.

Recent theoretical calculations of Pysh (47) predict that the positive dichroism of an antiparallel β structure should be split into two bands, both positive, centering at 197 and 190 nm. Our observations on poly-L-lysine in detergent solutions (40) lend some support to this belief, and indicate that the sharp asymmetrical drop of the difference curve of Fig. 5 seen below 197 nm is real. The uncertainties involved in the measurements of the various CD spectra in this range, however, make such detailed analysis unwarranted in the present case. Furthermore, it is known that aromatic residues also show optical activity in the lower wave length range (13, 14, 36, 37, 48, 49). The CD bands arising in this range overlap with bands characteristic of peptide bond transitions (33) and thus complicate further the analysis of CD spectra in terms of conformation. In the case of the β-lactoglobulins, the bands seen in the 270- to 300-nm region are weak (compare Figs. 1 and 2); this suggests that corresponding aromatic bands below 240 nm will not make major contributions to the over-all spectrum.

The circular dichroism data presented here underline the similarities between the various kinds of β-lactoglobulins rather than differences, which become emphasized when one deals with electrophoretic behavior (17, 18), titration (39), or amino acid composition (50, 51). All of the β-lactoglobulins of udder-bearing animals, as far as is known, are globular proteins of small axial ratio, dimers of identical single chain monomers

held together by noncovalent bonds, are reversibly dissociable at conditions of high net charge, and have dimer molecular weights of close to 36,000 (1, 50–55) or a bit less (56). That they are similar in many other respects can be inferred from the immunological studies of Johke, Hageman, and Larson (57), who found a marked cross-reaction between antisera to mixed β-A and β-B and the β-lactoglobulins of sheep, goat, and water buffalo, and from the report of Mawal, Barnabas, and Barnabas (58) who showed that β-B and buffalo β-lactoglobulin give identical tryptic fingerprints.

β-A, β-B, β-C, and G-β, with the exception of the small quantitative differences shown in Fig. 2, give circular dichroic spectra which are strikingly similar. G-β, as mentioned above, gives CD curves in this range almost superimposable on the β-B curve of Fig. 2, and β-A has a pH dependence of CD quite like the β-B data shown in Fig. 3 (40). Furthermore, all of the varieties tested show a practically identical α-helical pattern when dissolved in 99% acidic methanol (Fig. 4). In the region of aromatic chromophore absorption (Fig. 1A), β-A, β-B, and β-C do not differ sensibly.

An interesting inference may be drawn from the essential lack of any change in the CD patterns when the β-B (or β-A) is taken from isoelectric pH to pH 1. Under these conditions, the proteins are in monomer-dimer equilibrium (55) and, at 0.1 ionic strength, pH 1, and a concentration of 0.05% (a typical set of conditions for CD measurements in the ultraviolet), the fraction dissociated approaches 100%. The lack of any measurable change in the CD spectrum over this complete range of dissociation indicates that the secondary structures of the A and B variants and the mutual positions of their aromatic residues and disulfide bonds remain unchanged and unaffected by the presence of a considerable net positive charge ($\approx$20 per monomer at pH 1) on the molecule and the resulting strong electrostatic repulsive force.

The alkaline denaturation experiments shown in Fig. 3 also suggest strongly that certain portions of the β structure may be quite resistant to denaturation. In this connection, Gorbunoff (59) has found that one of the four tyrosines possessed by the β-A monomer does not become ionized at pH 12 and releases its proton only after 1 hour of exposure to pH 13. It seems plausible that this tyrosine is buried in the resistant β-structured region: the pH 7-insoluble material remaining when disulfide-cleaved β-lactoglobulins are digested with trypsin (60) contains tyrosine; this tryptic "core" was examined by infrared spectroscopy and found to consist predominantly of β-structured material.[8]

The CD data presented here confirm the earlier analysis (11) of the β-lactoglobulins as containing a small amount of α-helical conformation, almost 50% of unordered structure, and the remainder in an antiparallel β conformation. The actual percentages used in the calculations should be regarded only as working numbers. A further word of caution seems desirable in view of recent work by Greenfield, Davidson, and Fasman (61) and by Fasman and Potter (62). These authors have compared the ORD curves of lysozyme and myoglobin with curves calculated from spectra of poly-L-lysine in the α-helical, β-structured, and unordered conformations in the proper proportions, with the conclusion that such calculations tend to overestimate the amount of β structure present and to underestimate that of α-helix. Furthermore, the observation that films of polypeptides in various β structures may have quite

distinct ORD spectra (62) and CD spectra[9] additionally complicates any naive conformational analysis of a globular protein in solution. While present techniques make possible a qualitative examination of the structural composition of such proteins, future refinements in methods and availability of more models for the conformations considered should improve the accuracy of such analyses.

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